

Clinical Protocol: Summary Information

Clinical Protocol Title:

A double-blinded prospective randomized clinical trial comparing euploidy rates among embryos created from sibling oocytes exposed to sperm after ultrashort abstinence compared with standard abstinence

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1. Introduction

1.1 Background:

Current standard abstinence protocol:

Current standards for the duration of abstinence prior to a semen analysis are interpreted from the recommendations provided by the World Health Organization's laboratory manual for the examination and processing of human semen (WHO, 2021). In this manual, it is suggested that the most appropriate abstinence interval for semen analysis is 2-7 days, with the goal of achieving a more standardized period of abstinence in which to evaluate one semen sample to another (WHO, 2010). Though these recommendations have been adapted to the andrology and IVF laboratories, this period of abstinence does not inherently represent the most appropriate timing to achieve the highest quality semen with the best reproductive capability.

Rationale for short abstinence duration:

Efforts to improve semen quality, and thus reproductive outcomes, are at the forefront of IVF as male factor infertility may account for up to 30 percent of infertility. It has been shown that after a very short period of abstinence (within 4 hours) there are increased implantation rates, clinical pregnancy rates, and live birth rates (Barbagallo, 2023). It has been hypothesized that a

very short period of abstinence may improve sperm quality by reducing the time of transit through the epididymis leading to a shorter duration of exposure to reactive oxygen species and oxidative stress, epigenetic modifications, biochemical changes, and changes in seminal plasma composition that may adversely affect the reproductive capabilities of sperm (Barbagallo, 2023).

Embryologic, clinical, and genetic outcomes of sperm after use of short abstinence protocols:

There have been several studies analyzing the ways in which a shorter abstinence period may improve sperm DNA fragmentation, embryological, clinical, and genetic outcomes.

To address sperm DNA fragmentation, a prospective study involving men undergoing their first semen analysis in an infertility work up compared sperm DNA fragmentation in semen specimens after 3 days of abstinence and after 3 hours of abstinence and found that DNA fragmentation significantly improved in the 3 hour abstinence cohort ($p=0.0001$) (Dahan, 2021). This same study evaluated the semen parameters across the two groups and found that while semen volume and sperm concentration decreased, progressive motility tended to increase (Dahan, 2021). Similarly, a prospective cohort study including 52 men undergoing sperm DNA fragmentation analysis in addition to a standard semen analysis found that there were significant improvements in the sperm DNA fragmentation index in men providing a sample after 3-4 hours of abstinence compared with 2-5 days of abstinence (Karavani, 2023).

To address embryological and clinical outcomes, a prospective, sibling oocyte study analyzed 67 cycles using IVF and ICSI for oligoasthenozoospermic males to compare semen parameters and blastocyst formation for abstinence between 2-10 days and less than or equal to 3 hours (Li, 2023). They similarly found that short interval abstinence led to significantly improved sperm motility, morphological rate, and sperm DNA integrity before and after swim up (Li, 2023). The high quality blastocyst rate, available blastocyst rate, and oocyte utilization rates were all significantly higher in the short abstinence group with a trend towards higher clinical pregnancy, implantation, and live birth rates (Li, 2023). Adding to this knowledge, a meta-analysis analyzing the impact of the application of a very short abstinence period (within 4 hours) showed that a very short abstinence period significantly increased the implantation rate, clinical pregnancy rate, and live birth rate with assisted reproductive technology, primarily IVF with or without ICSI (Barbagallo, 2023).

To address genetic outcomes, a prospective, sibling oocyte trial analyzing 22 preimplantation genetic testing for aneuploid cycles using IVF and ICSI for oligoasthenozoospermic males compared 2-7 days of abstinence with 1 hour of abstinence for fertilization, blastocyst formation, ploidy rates, and clinical outcomes (Scarselli, 2019). This study found that semen volume was lower after shorter duration of abstinence with sperm concentration, motility and morphology similar between the groups (Scarselli, 2019). In regards to ploidy status, there was a higher blastocyst euploidy rate after the shorter duration of abstinence (Scarselli, 2019). However, this study was not randomized. This study concluded that prospective randomized controlled trials should be performed in order to confirm these findings (Scarselli, 2019).

1.2 Rationale:

As shown above, there is evidence that a short duration of abstinence can lead to improved embryologic, clinical, and genetic outcomes. These improved outcomes may be related to the improved sperm DNA fragmentation seen with a shorter duration of abstinence.

Though promising, prior studies have had limitations. Though there are some studies involving sibling oocytes, none include a prospective, randomized controlled trial and most have a limited number of cycles analyzed. Additionally, most studies include men with abnormal semen parameters requiring ICSI with limited studies evaluating the impact on men with normal semen parameters or standard insemination. Lastly, most studies use a swim up method of sperm preparation and do not include other methods of preparation such as density gradient centrifugation.

Currently, there are no randomized controlled trials evaluating euploidy rates in embryos created from exposure to sperm after the standard 2-5 days of abstinence compared to one hour of abstinence. The current study seeks to address each of the limitations above to contribute to and enhance the current literature regarding outcomes after shorter duration of abstinence.

2. Clinical Study Objectives

2.1 Primary Objective:

The primary aim of our study is to compare the euploidy rates between embryos created from sibling oocytes exposed to sperm after ultrashort abstinence with the use of ICSI.

We propose a double-blinded prospective randomized controlled trial to evaluate these embryologic outcomes. We hypothesize that there will be a higher euploidy rate among embryos created from the sibling oocytes exposed to sperm after ultrashort abstinence compared to standard abstinence.

2.2 Secondary objectives:

Secondary aims include comparing the euploidy rates between embryos created from sibling oocytes exposed to sperm with the use of standard insemination, semen parameters (volume, concentration, motility, morphology), fertilization rate, blastocyst rate, embryo quality, and total number of frozen embryos, pregnancy rates and miscarriage rates between the two groups.

3. Study Design

3.1 Overview of study design:

This is a double-blinded prospective randomized sibling oocyte study including patients undergoing treatment with IVF with standard insemination or ICSI at a single academic-affiliated IVF center (the Center for Advanced Reproductive Services).

Patients will be recruited at any time prior to or during their stimulation IVF cycle up until and including the day of trigger for participation in the study. Ovarian stimulation will occur utilizing protocols and doses set at the patients' physicians' discretion. During the oocyte retrieval, oocytes will be collected according to standard operating procedure.

The partner will produce and discard a sample 2 – 5 days prior to the day of oocyte retrieval. On the day of oocyte retrieval, they will produce two samples which will both be processed and used for the study. The first sample will be collected on the day of oocyte retrieval after 2 – 5 days abstinence. The second sample will be collected one hour after the first sample..

The oocytes will be exposed to the partners' sperm using either standard insemination or ICSI. The sperm will be prepared with the use of density gradient centrifugation.

For those oocytes undergoing standard insemination: After egg retrieval, all of the oocytes will be directly exposed to sperm. The oocytes will be randomized into one of two groups: group 1 (control group) and group 2 (study group). In group 1, oocytes will be exposed to sperm from semen samples collected after 2 – 5 days of abstinence. In group 2, oocytes will be exposed to sperm from semen samples collected after one hour of abstinence.

The andrologist will fulfill the randomization at the time of sperm preparation for either standard insemination or ICSI. Patients, physicians, and the embryologist performing the insemination will be blinded.

For those oocytes undergoing ICSI: After egg retrieval, the cumulus cells are removed. Once the cumulus cells are removed, the oocytes that are mature (metaphase II) at the time of ICSI will be randomized into one of two groups: group 1 (control group) and group 2 (study group). In group 1, oocytes will undergo ICSI utilizing sperm from semen samples collected after 2-5 days of abstinence. In group 2, oocytes will undergo ICSI utilizing sperm from semen samples collected after one hour of abstinence. Embryos resulting from both groups will be cultured for 5-7 days. High-quality blastocysts will be biopsied for genetic analysis via preimplantation genetic testing (PGT) on day 5, 6, or 7 after fertilization.

Euploid embryos will be transferred in a subsequent frozen embryo transfer (FET) cycle. Endometrial preparation protocols will be chosen, again, at the patients' physicians' discretion. The patient's morphologically best euploid embryo will be selected by embryology lab staff for embryo transfer in this cycle. After transfer of this embryo, if the patient becomes pregnant, then pregnancy outcomes will be followed until discharged to her obstetrician.

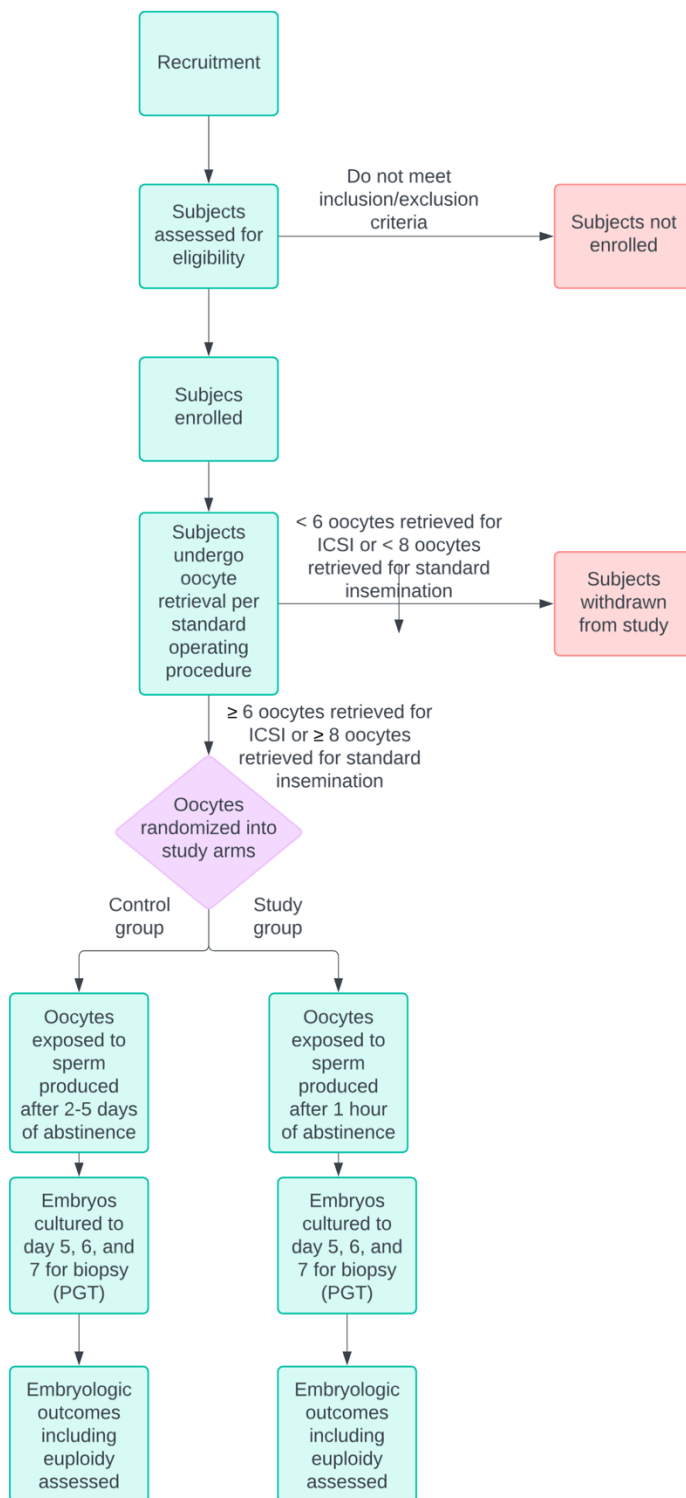
The IVF operational lab director will rank the embryo morphology for any patient with multiple euploid embryos and will be blinded as to which oocytes were randomized to each group during the stimulation cycle and as to which embryos were in each group. The embryologist ranking embryos by quality will also be blinded and will choose the best embryo to transfer utilizing PGT results as well as morphology regardless of the sperm selection method used. Therefore, none of these parties will be able to influence which embryo to transfer based on study group.

A sibling oocyte study is a study in which the oocytes from each IVF cycle are separated into two groups. This is the ideal approach to study this abstinence protocol, as oocytes exposed to the same stimulation environment can be randomized to both the control and study groups. The prospective evaluation of sibling oocytes undergoing insemination with sperm derived from 2-5 days compared with one hour of abstinence can be used to study the semen parameters, fertilization rates, blastulation rates, and euploidy rates when implementing this new abstinence protocol in the IVF laboratory.

The expected total duration of subject participation will include the duration of the patient's stimulation IVF cycle (approximately 30 days), the duration of the patient's first FET cycle (approximately 30 days), and early pregnancy monitoring, which is the same as non-study participants. If a subject were to conceive in the FET cycle, then participation would continue with serum steroid monitoring until the patient is discharged from our practice to their chosen obstetrician. This gives a total non-consecutive participation time of approximately 60 days for patients who do not conceive, and up to 12 additional weeks if they do conceive in their FET cycle.

3.2 Study Design Schematic:

3.2.1 Stimulation cycle schematic



4. Statistical Methods/Data Analysis

4.1 Study endpoints:

4.1.1 Primary endpoint

The primary aim of our study is to compare the euploidy rates between embryos created from sibling oocytes exposed to sperm produced after 2-5 days of abstinence compared with 1 hour of abstinence using ICSI.

Euploidy rate: defined as the number of euploid embryos in each group divided by the total number of mature oocytes

Number of mature oocytes: defined below for ICSI and standard insemination respectively

Maturity for patients undergoing ICSI is determined on the day of oocyte retrieval. Upon removing the cumulus, maturity is defined by the presence of a polar body within the oocyte (metaphase II).

Maturity for patients undergoing standard insemination is determined the day after oocyte retrieval (Day 1). On Day 1, the maturity of the oocytes is determined after removal of residual cumulus cells. Maturity is defined by the presence of a polar body within the oocyte (metaphase II).

4.1.2 Secondary endpoints

The main secondary aim is to determine if ultrashort abstinence protocol improves euploid rates in women using standard insemination with normal semen parameters. Other secondary aims include comparing the semen parameters (volume, concentration, motility, morphology), fertilization rate, blastocyst rate, embryo quality, and total number of frozen embryos between the two groups.

- Semen volume: defined as the amount of semen produced (mL)
- Sperm concentration: defined as the number of sperm per unit volume of semen (millions/mL)
- Sperm motility: defined as the percentage of sperm that is progressively moving forward
- Sperm morphology: defined as the percentage of sperm with a normal appearance
- Fertilization rate: defined as the number of oocytes that fertilized normally (as evaluated by the embryologists' visualization of two pronuclei on the day after insemination) divided by the number of mature oocytes retrieved
- Blastocyst utilization rate: defined as the number of top quality blastocysts biopsied and frozen divided by the number of oocytes with normal fertilization
- Day 5 blastocyst utilization: defined as the number of top quality blastocysts biopsied and frozen on day 5 divided by the number of oocytes with normal fertilization
- Total number of frozen embryos: defined as the total number of blastocysts biopsied and frozen in each group

- Embryo quality: defined by the Gardner scale and compared between both groups (Gardner, 2000)
- Pregnancy rate: defined as the number of pregnancies as determined by positive pregnancy test 9 days after embryo transfer divided by the number of patients undergoing transfer
- Clinical pregnancy rate: defined as the number of patients with at least a gestational sac on ultrasound divided by the number of patients undergoing embryo transfer
- Miscarriage rate: defined as the number of pregnancies ending in losses (biochemical [no pregnancy seen on ultrasound] or clinical [pregnancies with at least a gestational sac visualized on ultrasound]) divided by the number of pregnancies
- Ongoing pregnancy rate: defined as the number of pregnancies with a gestational sac, yolk sac, fetal pole and fetal heart rate that are ongoing at the time of discharge from our practice divided by the number of patients undergoing embryo transfer.

4.2 Sample size determination:

Based on a 0.05 two-sided significance level, enrollment ratio of 1:1, and a baseline euploidy rate per mature oocyte retrieved of 10% at our center (Thorne, 2019), we calculated that a sample size of 686 oocytes in each group for a total of 1372 oocytes will provide 80% power to detect a significant difference in the euploidy rates per mature oocyte between the study group proportion of 0.15 and the control group proportion of 0.10. This difference is similar to a sibling oocyte study using ultrashort abstinence (Scarselli et al, 2019). Allocating for a participant dropout of approximately 10%, we intend to enroll up to 187 patients. As our sample size is not based on participants recruited but rather on number of oocytes randomized, a specified sample size of 1372 oocytes should be adequate to reach our goal sample size. Since there has not been any previous study using ultrashort protocol in standard insemination cycles, and because about 25% of patients utilize standard insemination in our practice, we estimate that an additional 343 oocytes resulting in a total of 1715 oocytes will need to be randomized. Therefore, we intend to recruit a total of 187 patients. This sample size will be feasible to obtain. Our IVF center performs over 1,000 oocyte retrievals per year, with about 6% of those utilizing PGT (unpublished internal review data).

4.3 Programs to be used for data analysis:

Statistical analyses will be performed using Statistical Package for the Social Sciences (SPSS, version 26, Chicago, IL). McNemar's test will be used for paired analysis of categorical variables. A paired t-test will be used for paired normally distributed continuous variables and a Wilcoxon test will be used for paired non-parametric continuous variables where appropriate. Further analysis will stratify euploidy rates by male semen parameters (normal or abnormal) and insemination method (standard insemination or ICSI) for the study group and control group.

5. Subject Selection

5.1 Subject inclusion criteria:

All patients recruited in the study must be undergoing IVF treatment at the Center for Advanced Reproductive Services. Subjects may be recruited by their physician at any office visit prior to IVF stimulation start, at their visit for baseline ultrasound of their IVF cycle, or at any office visit prior to and including the day of trigger. Up to 187 subjects will be enrolled in this study.

Subjects must meet all of the following inclusion criteria to be recruited into the study:

- Subjects are nonpregnant females ≥ 18 years and ≤ 42 years of age.
- Subjects obtain ≥ 6 mature oocytes at the time of oocyte retrieval for ICSI or ≥ 8 oocytes at the time of oocyte retrieval for standard insemination.
- Subjects are utilizing standard insemination or ICSI for fertilization.
- Subjects are undergoing PGT-A (PGT for aneuploidy) or PGT-M (PGT for monogenic disorders).
- Subjects are willing to comply with study protocol and procedures and provide written informed consent.

5.2 Subject exclusion criteria:

Subjects will be excluded from the study if they meet any of the following criteria:

- Subjects are utilizing donor oocytes, donor sperm, or gestational carrier.
- Subjects have a diagnosis of cryptozoospermia (no spermatozoa identified in fresh semen sample).
- Subjects are utilizing surgically removed sperm (e.g. via testicular sperm aspiration [TESA] or microsurgical epididymal sperm aspiration [MESA]).
- Subjects are utilizing frozen/thawed sperm, including in cases in which a fresh sample was planned and the sample is insufficient for use.
- Subjects are utilizing frozen/thawed oocytes.
- Subjects undergoing PGT-SR (PGT for structural rearrangements).
- Subjects are undergoing a day 3 (cleavage stage) embryo transfer.
- Subjects obtain < 6 mature oocytes at the time of oocyte retrieval for ICSI or < 8 oocytes at the time of oocyte retrieval for standard insemination.
- Subjects obtain ≥ 6 mature oocytes at the time of oocyte retrieval for ICSI or ≥ 8 oocytes at the time of oocyte retrieval for standard insemination but choose to fertilize fewer than 6 (for ICSI) or 8 (for standard insemination).
- Male partner is unable to produce a second semen sample within three hours of the first semen sample (standard abstinence sample)
- Male partner has an infectious disease.

Selection of patients into the study will be performed by the physicians, study coordinator principal investigator, or trained consenters. The selection criteria outlined above was established based on clinical experience and quality control data from CARS. This data indicates that patients over the age of 42 have a poorer prognosis and are less likely to develop blastocysts in culture. This patient population may benefit from a transfer at the cleavage stage and therefore would not be an appropriate group to include in this study. The use of donor oocytes, thawed

oocytes, and surgically retrieved sperm would introduce confounding variables that could affect the validity of the results and therefore have been listed as exclusion criteria.

For those with a plan to undergo standard insemination, if the second (ultrashort abstinence) sample produced on the day of oocyte retrieval is not adequate for standard insemination, the first (standard abstinence) sample will be utilized and standard insemination will be performed with that sample.

On the day of oocyte retrieval, subjects will not have their oocytes randomized if less than 6 mature oocytes are retrieved for the ICSI group or if less than 8 oocytes are retrieved for the standard insemination group. Subjects may be withdrawn if the sperm sample parameters are too low for ICSI on the day of oocyte retrieval as it will not be possible to perform ICSI and they may require use of a frozen sample as outlined in the exclusion criteria.

Subjects will be withdrawn if they or their physician opts for a cleavage stage embryo transfer or to forego the use of PGT testing during their cycle, or if the subject voluntarily declines to participate after enrollment. If the participant is withdrawn or voluntarily withdraws from the study sperm will be processed via density gradient centrifugation and insemination will be performed with standard insemination or ICSI as determined by the patient and physician.

6. Research Study Procedures

6.1 Screening, recruitment, and consenting procedures:

All new or established patients undergoing IVF treatment at the Center for Advanced Reproductive Services will be considered for inclusion in the study.

Electronic flyers will be displayed on social media outlets as well as the Center for Advanced Reproductive Services website. Physical flyers will be displayed in the Center for Advanced Reproductive Services (all locations including satellite locations).

Eligibility for inclusion will be determined at the time of their visit with their physician based on their conversation during their routine care. If eligible and interested in the study, verbal permission will be obtained from the patient to be contacted by the study team.

The physicians of patients who express interest in participating in the study will contact the CARS research office to alert them of a potential participant. The research team will not contact patients to inquire about their interest in the study unless verbal permission has been obtained from the patient to be contacted by the study team.

The patients will be contacted via email by a member of our research staff. In this email, more information about the study will be given to potential participants, including the study consent form to review. Subjects will also receive a phone call to review all study information and to have the opportunity to have their questions answered.

Subjects who are eligible and agree to participate will be consented for the study at any office visit, over the phone, or through a telehealth visit through a virtual platform at any time up to the day of trigger of oocyte maturation, as outlined below. We are asking for an exemption to the consenting witness requirement because our standard practice for routine appointments has transitioned to a HIPAA compliant telehealth visit after the COVID-19 pandemic and we are applying these policies to consenting for this study.

The research staff will review the informed consent form (either in person, by phone or via telehealth) with the potential participants and address any questions or concerns, and the potential participant will be given ample time to consider the decision to participate prior to signing the consent form. If potential participants will be signing consents in person, they will be moved into a private room if not already in a private area for the consent to be reviewed and all questions to be answered. The length of time needed for discussion at the time of consenting will be approximately 10 minutes, as the patient and partner will have reviewed the information ahead of time. The patient will be encouraged to have her partner present at the time of consenting so that both may sign the consent form on the same day. However, if the partner is not available to sign on that day, then they may sign the consent in any of the three ways outlined above at any time up until and including the day of the oocyte retrieval.

After the participant has been told about the study and had time to consider the decisions, consent will be documented in one of the following ways:

- In person, with the CARS staff and the participant both signing the same consent form. A copy of the signed consent will be provided to the participant.
- Over the phone or via telehealth, with the CARS staff and the participant signing separate hard copies of the consent form at the same time while both on the phone. These two forms will then be stapled together upon the participant's next visit. A copy of the signed consents will be provided to the study subject.
- E-signing an electronic consent using eSign (EngagedMD) with the CARS staff. The CARS representative who previously discussed the consent form with the patient via telephone subsequently will attest to the documentation of consent electronically and the signed consent will be available to the participant through an automated email representing a completed copy of the fully executed consent.

6.2 Study procedures:

Subjects participating in this study will proceed with their IVF cycle according to clinical standard operating procedure. All procedures, including blood and ultrasound monitoring, dose adjustment, oocyte retrieval and embryo transfer will take place according to the patient's clinical treatment plan at their physician's discretion.

For oocytes undergoing standard insemination: On the day of vaginal oocyte retrieval, oocytes collected from the patient will be exposed to sperm. These oocytes will be randomized as described below (see section 6.3). Patients' partners will provide two semen samples on the morning of the oocyte retrieval to use for standard insemination as described below. A small aliquot (5-10 ul) will be taken from each to assess semen parameters. The remaining sample

volumes will be prepared over a gradient. After the procedure, the final samples will be frozen for subsequent analyses (see section 6.4 below). As part of the procedure for assessing fertilization, the oocytes will simultaneously be assessed for nuclear maturation the day after VOR.

For oocytes undergoing ICSI: On the day of vaginal oocyte retrieval, oocytes collected from the patient will be stripped using hyaluronidase and assessed for nuclear maturation prior to ICSI by one of the embryologists. These oocytes will be randomized as described below (see section 6.3). Patients' partners will provide two semen samples on the morning of the oocyte retrieval to use for ICSI as described below. A small aliquot (5-10 ul) will be taken from each to assess semen parameters. The remaining sample volumes will be prepared over a gradient. After the ICSI procedure, the final samples will be frozen for subsequent analyses (see section 6.4 below).

Oocytes from Group 1 will undergo standard insemination or ICSI with sperm from semen samples collected after 2-5 days of abstinence and processed using density gradient centrifugation methods. Oocytes from Group 2 will undergo standard insemination or ICSI with sperm from semen samples produced after 1 hour of abstinence and processed using density gradient centrifugation methods. This method of sperm processing and these procedures will be the same as all patients undergoing standard insemination or ICSI at our Center and are described in detail below.

Fresh ejaculate will be incubated at room temperature to allow for liquefaction. The semen sample volume will be measured using a serological pipette, and characteristics of the semen sample will be noted on the sperm preparation report, including abnormal findings. Pre-preparation mean counts and motility will be noted on the sperm preparation data sheet. The sample is pipetted into two tubes of 90% gradient and then centrifuged. The centrifuge pellet is then transferred into sperm wash media and the post-preparation count and motility is recorded. The sample is then centrifuged again, and the centrifuge pellet is then underlaid with 0.5mL of Global Fert Media. This sample is then incubated at 37°C and the motile sperm are allowed to swim up into the supernatant.

Subjects using adjuncts for sperm selection including microfluidics and PICS (physiologic ICSI) will be eligible for the study.

For standard insemination, the oocytes are exposed to sperm. The oocytes will be evaluated 16-18 hours later for evidence of fertilization (day 1 after oocyte retrieval and insemination).

For ICSI, a small aliquot of the supernatant is added to approximately 10 µL of 7% polyvinylpyrrolidone (PVP) and visualized under a microscope, at which point individual sperms for ICSI are selected from this sample by the embryologist. Injected oocytes will be evaluated 16-18 hours after the ICSI procedure for evidence of fertilization (day 1 after oocyte retrieval and insemination).

All embryos will be cultured in the same dishes, single-step culture media, and incubators as is currently used for all patients undergoing IVF in our clinic and will undergo monitoring at the same checkpoints.

Embryologic outcomes in the resulting embryos will be observed on day 5 after VOR. On day 5 after VOR, embryos will be visualized and graded according to a Gardner scale (Gardner, 2000). If an embryo reaches grade B3BB or better on the Gardner scale, then this embryo will undergo trophectoderm biopsy and will subsequently be frozen via vitrification. In this lab, the zona is breached using the laser at the same time as the biopsy procedure and therefore the TE does not herniate out of the zona prior to biopsy. The same procedure will be followed on day 6 and day 7 for blasts that reach grade B3BB or better on these days.

After cryopreservation and at the completion of the cycle, the operational lab director (who will be blinded to treatment arm) will establish a rank for selection purposes according to morphology. Standard laboratory procedure includes the primary consideration of the degree of blastocyst expansion on Day 5, followed by the score of the inner cell mass (ICM) and trophectoderm (TE) in that order.

PGT results will be sent to the PGT lab for genetic analysis. Any euploid and mosaic embryos will be kept in storage, while any aneuploid embryos will be discarded. If there are multiple euploid embryos to choose from for the subsequent FET cycle, a single euploid embryo will be selected according to the pre-determined rank, established previously and blinded to the study group. If there are no euploid embryos for transfer, the patient's participation in the study will end at this point.

In the patient's FET cycle, endometrial preparation and embryo transfer will occur according to standard procedure and the patient's physician's discretion (Toth, 2017, Godiwala, 2022). Only one euploid embryo will be transferred according to American Society for Reproductive Medicine (ASRM) guidelines (Penzias, 2017).

Subjects undergoing embryo transfer will be followed until pregnancy outcome has been determined (i.e. date of negative hCG test or clinical pregnancy ultrasound if pregnant). Patients will be followed utilizing standard serum hormone levels as well as ultrasounds in our practice until the patient is discharged to their chosen obstetrician.

6.3 Randomization and allocation to treatment:

Eligible participants will be consented and enrolled up to and including the day of trigger. Recruitment will occur at the time of the consultations with the physicians or at any point during the patient's IVF cycle up to and including the day of trigger. The eligibility assessment, consenting and enrollment will be performed by the physician, principal investigator, study coordinator, or trained consenters. Consented eligible study participants with ≥ 6 mature oocytes at the time of oocyte retrieval for ICSI or ≥ 8 oocytes at the time of oocyte retrieval for standard insemination will have their oocytes randomized in a 1:1 ratio into either the control or study arm

on day 0 (the day of oocyte retrieval). Consented patients with less than 6 mature oocytes will be excluded from the study.

The oocytes will be exposed to sperm through either standard insemination or ICSI. The oocytes undergoing standard insemination will be separated into two cohorts and exposed to sperm with the number of mature oocytes determined by the number of oocytes displaying a polar body on observation on day 1. The oocytes undergoing ICSI will be treated with hyaluronidase to remove the cumulus cells, and then at the time of ICSI, the mature oocytes will be identified and separated into two cohorts by the embryologist. The cohorts will be group A (alpha) and group B (beta).

The oocytes will be randomly assigned to one of the two groups in a 1:1 ratio. A computerized randomization program will be used to assign group A as either sperm produced after standard abstinence or ultrashort abstinence. The remaining group of oocytes (group B) will then be randomized into the opposite group.

6.4 Secondary sperm analysis:

The remainder of the samples from both the standard abstinence and ultrashort abstinence groups will be frozen for sperm DNA fragmentation and DNA methylation studies. Hypotheses for improved outcomes with shorter durations of abstinence include decreased transit time through the epididymis which may lead to less exposure to oxidative stress and less DNA fragmentation (Barbagallo, 2023). Previous research has indicated that shorter duration of abstinence leads significantly improves sperm DNA fragmentation (Dahan, 2021). Likewise, it has been hypothesized that there may be a difference in the metabolic profile, including the amount of pyruvate and taurine, of seminal plasma after a shorter duration of ejaculation (Alipour, 2021). In addition to sperm DNA fragmentation, sperm DNA methylation and epigenetic changes have been proposed to contribute to male factor infertility (Lujan, 2019).

Sperm DNA fragmentation analysis tests the integrity of the DNA only; no genetic testing of chromosomes or DNA will occur. This analysis will be performed by ReproSource. Sperm DNA methylation analysis tests genome wide alterations in sperm DNA methylation. This analysis will be performed by a SpermQT test through Path Fertility.

Vials will be frozen and stored in a de-identified fashion and will be discarded after the DNA fragmentation and epigenetic studies are complete. Patients will be consented for this additional analysis at the time of consent for this study and will be able to opt out of freezing this sperm sample if desired.

The remaining sperm sample volume after processing must be at least 100 µl to freeze a leftover sample. If the remaining sperm sample volume is less than 100 µl, randomization will occur, but freezing of sperm samples for future analysis will not occur as this volume is too low to allow for analysis.

6.5 Specimen processing:

Semen analyses, including concentration, motility, morphology, liquefaction, viscosity, pH, round cell count, and volume will be conducted in the laboratory at the Center for Advanced Reproductive Services. Secondary semen analyses, such as sperm DNA fragmentation and epigenetics, will be outsourced for processing. The DNA fragmentation analyses will be performed by ReproSource. The sperm epigenetic analyses will be performed by a SpermQT test through Path Fertility. The samples will be sent frozen to the respective companies as coded samples.

7. Safety and Effectiveness Assessments

7.1 Safety assessments:

This study does not involve any interventions or changes to standard procedure and therefore there are no additional risks to the subjects or the embryos. Risks of COH include ovarian hyperstimulation syndrome, risks of anesthesia, and the possibility that no oocytes will be retrieved. Risks of embryo culture and biopsy include the possibility that no embryos make it to the blastocyst stage or that no embryos are euploid. All of these risks will be discussed with the patient ahead of time, will be reviewed in the standard CARS consent forms signed by the patient, and will be monitored according to standard operating procedure at CARS.

7.2 Adverse events:

All observed adverse events, defined as any unexpected negative occurrence associated with participation in the study, as well as any abnormal findings even if not associated with study participation, will be recorded in the subjects' records. For all adverse events, sufficient information will be pursued and or obtained so as to permit an adequate determination of the outcome of the event and assessment of the causal relationship between the adverse event and study participation. Adverse events or abnormal test findings felt to be associated with participation in the study will be followed until a resolution is reached.

In accordance with the applicable policies of the University of Connecticut Health Center Institutional Review Board (IRB), the investigator will report to the IRB any observed adverse event that is determined to be associated with participation in the study. Applicable adverse events will be reported to the IRB as soon as possible and no later than 7 calendar days following the investigator's receipt of the respective information. Follow up information to a reported adverse event will be submitted to the IRB as soon as the relevant information is available.

8. Data Safety Monitoring Plan

8.1 Summary of Data Safety Monitoring Plan:

The Data Safety Monitoring Plan (DSMP) for this study will include an annual meeting to review quality control measures and study outcomes. The outcomes specified in the Appendix B form will be reported on an annual basis after each DSMP meeting. Briefly, these measures

include serious adverse events, a summary of all adverse events, protocol deviations, interim analysis of outcome data, confirmation of data integrity and confidentiality, product accountability, and study documentation. Only unblinded data will be reviewed.

While this is an investigator-initiated interventional trial, the intervention is not performed on participants themselves, but rather on the oocytes and sperm produced, therefore making this a minimal risk study to participants. Because of this, a Data Safety Monitoring Board (DSMB) is not required and the measures proposed will be reviewed annually at a meeting led by the PI and study staff. If it is determined that the study will be suspended for any reason, new subjects will not be enrolled but the continued monitoring of previously enrolled subjects will occur.

9. Data Handling and Record-Keeping

9.1 Data collection:

The medical record will be queried for each subject enrolled into the clinical study. A detailed list of the data collected for the study is outlined in the data collection form. Data collected will consist of the following:

- Patient demographics including age, parity, body mass index (BMI), infertility diagnoses, prior use of PGT, type of PGT being utilized, reason for PGT utilization, and baseline hormone levels (baseline FSH, LH, E2, AMH).
- Patient stimulation cycle information including IVF cycle number, stimulation protocol, gonadotropin dosages, days of stimulation, peak estradiol levels, follicular measurements, and trigger medication and dosages.
- Number of oocytes retrieved, number of mature oocytes, and number of oocytes allotted to either the study group or the control group.
- Partner age, post-processing volume, concentration, motility, morphology in both the control (2-5 days of abstinence) and experimental (1 hour of abstinence) groups, duration of abstinence.
- Fertilization results, including normal fertilization, abnormal fertilization, and count of embryos after VOR.
- Embryo biopsy data in both control and study groups, including number of embryos biopsied/frozen and results (euploid, aneuploid, mosaic, and no result).
- Frozen embryo transfer cycle information including IVF cycle number, endometrial preparation protocol, number of embryos transferred, identification number of embryo(s), transferred, whether the embryo(s) transferred were from the study or control group, and morphology data from assessments at the time of embryo transfer.
- Results of serum pregnancy tests, including final outcome (miscarriage, ectopic pregnancy, clinical pregnancy, ongoing pregnancy), number of gestational sacs, initial pregnancy ultrasounds, and whether the patient delivered

Data will be collected into the medical record, which will then be queried and copied directly onto the data collection form by the designated co-investigators. No data collected for this study is additional to that which is already collected for clinical purposes.

If data is missing from the data collection form, a designated co-investigator will review the source data to determine if the data is present and will accurately complete the data collection form. If data is unable to be obtained it will be reported as missing.

9.2 De-identification of data:

Subject-specific data will be coded. The code will consist of a 2-letter study abbreviation followed by a sequential 3-digit number. A subject identification code list will be stored and secured in a password protected research folder. The randomization list will also be stored and secured in a password protected research folder. These research records will be only accessible by study personnel. Subject names or other directly identifiable information will not appear on any reports, publications or other disclosures of clinical study outcomes. Databases and study spreadsheets will be stored electronically in password protected files managed by the study coordinator, co-investigators and principal investigator.

The research findings will be submitted for publication at the culmination of the study. Subjects will not be identified and confidentiality will be maintained. The subjects will not be notified of the research findings.

9.3 Record Maintenance and retention:

The principal investigator will maintain records in accordance with Good Clinical Practice guidelines; to include:

- IRB correspondence related to the protocol including copies of adverse event reports and annual or interim reports
- Current and past versions of the IRB-approved protocol and corresponding IRB-approved consent forms and subject recruitment advertisements
- Financial disclosure information
- Curriculum vitae
- Certificates of required training
- Signed informed consents
- Monitoring visit reports
- Subject enrollment logs
- Subject identification code list
- Master randomization list
- DSMP report

The principal investigator will retain the specified records and reports for up to 2 years after the completion of the study.

10. Ethics

10.1 Institutional Review Board (IRB) approval:

The Sponsor-Investigator will obtain, from the UConn Health Institutional Review Board (IRB), prospective approval of the clinical protocol and corresponding informed consent form(s); modifications to the clinical protocol and corresponding informed consent forms, and advertisements (i.e., directed at potential research subjects) for study recruitment.

The only circumstance in which a deviation from the current IRB-approved clinical protocol/consent form(s) may be initiated in the absence of prospective IRB approval is to eliminate an apparent immediate hazard to the research subject(s). In such circumstances, the Sponsor-Investigator will promptly notify the UConn Health IRB of the deviation.

The UConn Health IRB operates in compliance with FDA regulations at 21 CFR Parts 50 and 21 CFR 56, and in conformance with applicable International Conference on Harmonization (ICH) Guidelines on Good Clinical Practice (GCP).

In the event that the UConn Health IRB requires, as a condition of approval, substantial changes to a clinical protocol submitted, or in the event of the Sponsor-Investigator's decision to modify the previously accepted clinical protocol, the Sponsor-Investigator will submit (i.e., in advance of implementing the change) a Protocol Amendment.

10.2 Ethical and scientific conduct of the clinical research study:

The clinical research study will be conducted in accordance with the current IRB-approved clinical protocol; ICH GCP Guidelines adopted by the FDA; and relevant policies, requirements, and regulations of the UConn Health IRB, University of Connecticut and UConn Health, state of Connecticut, and applicable federal agencies.

10.3 Subject informed consent:

The Sponsor-Investigator will make certain that an appropriate informed consent process is in place to ensure that potential research subjects are fully informed about the nature and objectives of the clinical study, the potential risks and benefits of study participation, and their rights as research subjects. The Sponsor-Investigator, or a sub-investigator(s) designated by the Sponsor-Investigator, will obtain the written, signed informed consent of each subject prior to performing any study-specific procedures on the subject. The date and time that the subject signs the informed consent form and a narrative of the issues discussed during the informed consent process will be documented in the subject's case history. The Sponsor-Investigator will retain the original copy of the signed informed consent form, and a copy will be provided to the subject.

The Sponsor-Investigator will make certain that appropriate processes and procedures are in place to ensure that ongoing questions and concerns of enrolled subjects are adequately addressed and that the subjects are informed of any new information that may affect their decision to continue participation in the clinical study. Patients and their partners will be consented by CITI-trained study consenters at any time before or during their IVF cycle up to and including the day of trigger. The investigator will make certain that appropriate processes and procedures are in place to ensure that ongoing questions and concerns of enrolled subjects are adequately addressed and that the subjects are informed of any new information that may

affect their decision to participate in the study. In the event of substantial changes to the clinical study or the risk-benefit ratio of study participation, the investigator will obtain the informed consent of enrolled subjects for continued participation in the study.

10.4 Subject compensation:

Enrolled study participants that complete the study requirements will not receive any monetary incentive for their participation.

11. Study Discontinuation Criteria

11.1 Discontinuation of individual research subjects:

As described in prior sections should a subject have an unrelated serious adverse event requiring hospitalization in which monitoring could not be performed that subject would be withdrawn from the study. Data would be collected and placed in the research record up until the time of the withdrawal. The withdrawn subject would be reported in the final analysis. Monitoring would continue to ensure patient safety. Research subjects may also voluntarily withdraw from the study without affecting their clinical care.

11.2 Sponsor-Investigator discontinuation of the clinical research study:

If at one of the scheduled reviews, it is determined that there is a significant detrimental effect of the study and that the continuation of the study will put the participants at significant risk, the study will be discontinued. If new information is published in the literature that will suggest that continuation of the study will pose significant risks to the participants, the study will be discontinued. If the study is discontinued the UConn Health IRB will be notified promptly of discontinuation of the study.

12. Budget

12.1 Funding source:

No outside funding has been obtained for this study.

13. References for protocol

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